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16. ABSTRACT

Neuropathological and biochemical effects of neonatal exposure to the alkyl metal triethyltin were examined in Long Evans juvenile male rats. Rats were injected intraperitoneally on post-natal day 5 with 6 mk/kg of triethyltin bromide and sampled on day 20. The brains of tin-treated animals weighed significantly less than either saline or starved controls and exhibited a marked anterior-posterior atrophy. Histologically, neuronal necrosis was noted in the enthorhinal and transitional cortex, an observation confirmed by immunocytochemical staining of astrocytes. Hippocampal involvement was further evidenced by a protrusionof the molecular layer of the dentate gyrus, and an abnormal staining pattern of acetylcholinesterase in this layer. Sections stained by the Timm's method for the deposition of heavy metals showed a marked reduction in the staining of the hippocampal CA4-2 sectors and an absence of stained laminae in the outer molecular layer of the dentate gyrus. Receptor binding assays indicated a selective depression of the benzodiazepine receptor in the hippocampus of tin-treated pups compared to starved controls. Taken in concert, these data indicate that neonatal exposure to triethyltin produces severe neuronal damage in the posterior cortex and a derangement of hippocampal afferent circuitry.

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Triethyltin-Induced Neuronal Damage in Neonatally Exposed Rats

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ABSTRACT: *Neuropathological and biochemical effects of neonatal exposure to the alkyl metal triethyltin were examined in juvenile male Long Evans rats. Rats were injected intraperitoneally on postnatal day 5 with 6 mg/kg of triethyltin bromide and sampled on day 20. The brains of tin-treated animals weighed significantly less than either saline or starved controls and exhibited a marked caviation of the ventrolateral surfaces. Histologically, neuronal necrosis was noted in the entorhinal and transitional cortex, an observation confirmed by immunocytochemical staining of astrocytes. Hippocampal involvement was further evidenced by a protrusion of the molecular layer of the dentate gyrus, and an abnormal histochemical staining pattern of acetylcholinesterase in this layer. Sections stained by the Timm's method for the deposition of heavy metals showed a marked reduction in the staining of the hippocampal CA4,3,2 sectors and an absence of stained laminae in the outer molecular layer of the dentate gyrus. Receptor binding assays indicated a selective depression of the benzodiazepine receptor in the hippocampus of tin-treated pups compared to starved controls. Taken in concert, these data indicate that neonatal exposure to triethyltin produces severe neuronal damage in the posterior cortex and a derangement of hippocampal afferent circuitry.*

Key Words: Triethyltin, Neuronal Neurotoxicity, Postnatal Toxicity

INTRODUCTION

Triethyltin (TET) is a classic myelinotoxicant which produces severe central nervous system (CNS) edema in the adult after acute exposure (Magee *et al.*, 1957; Aleu *et al.*, 1963; Watanabe, 1981). Biochemical and morphological studies indicate that the myelin sheath is the primary target tissue (Locke and Aldridge, 1975; Jacobs *et al.*, 1977). Although the underlying biochemical lesion is presently unresolved, several possibilities have been proposed involving inhibition of Na^+/K^+ ATPase, (Torak 1965; Jacobs *et al.*, 1983), impairment of energy related metabolism, specifically glucose oxidation (Cremer 1970; Locke, 1976), and uncoupled mitochondrial oxidative phosphorylation (Aldridge, 1958; Stockdale, *et al.*, 1970). A variety of pathogenic changes results from

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acute or chronic neonatal exposure to the alkyl metal including hemorrhagia, cellular necrosis and myelin edema (Suzuki, 1971; Watanabe, 1977). In addition, disruption of myelinogenesis (Blaker *et al.*, O'Callaghan *et al.*, 1983), behavioral (Reiter *et al.*, 1981; Harry and Tilson, 1981) and electrophysiological (Dyer *et al.*, 1981) alterations have been demonstrated. In this study, using conventional and histochemical light microscopic techniques and receptor binding assays, we have documented TET-induced neuronal alterations in the posterior cortex of neonatally exposed juvenile rats.

EXPERIMENTAL PROCEDURES

Animals

Randomly selected Long-Evans, male pups (n=60) (Charles Rivers, Portage, MI.) were injected intraperitoneally on postnatal day (PND) 5 with 6 mg/kg TET-bromide (Alfa Chemicals, Danvers, MA) or phosphate-buffered saline vehicle in a volume of 10μl/gm. Starved controls were also included in our study to provide body weight-corrected samples. For this, saline-treated pups were separated from their mother for 8 hrs and then returned to oversized litters (n=16). This procedure was repeated for 3d which approximated the length of time TET-treated pups showed a reduced or absent milk band. Eight litters, composed of TET (n=3), starved controls (n=3) and saline controls (n=2), were used for histological examination and brain weight data. For receptor binding assays, 12 randomly selected TET-treated pups (n=6) and starved controls (n=6) were used.

Pathology

Tissue Preparation: Whole litters (n=8) were killed at PND-20. Animals were weighed, anesthetized with Chloropent containing 1% heparin (50 units/ml) and perfused through the heart with 3.0% Sorensen's-buffered glutaraldehyde. The brains were allowed to fix *in situ* overnight at 4°C before being removed from the cranial cavity. After gross examination, brains were sectioned in the mid-horizontal plane to give an overview of the entorhinal cortex (ERC) and hippocampus.

Histology: Paraffin-embedded tissues, cut on a rotary microtome at 6-8 microns, were stained with either 1% cresyl violet acetate or hematoxylin-eosin. Sixteen (TET=6n; starved=6n; saline=4n) pups were randomly selected from 8 liters and furnished tissue for each of the following histochemical procedures: the Timm's stain for heavy metal deposition (Danscher, 1981); a modified Friedenwald-Koelle technique for acetylcholinesterase (Lynch *et al.*, 1972) and the Rapid Golgi stain for metal impregnation of the neuronal cell body and dendrites (Kemali, 1976). In addition, 8 pups were reserved for a commercially obtained (Dako Corp., Santa Barbara, CA.) peroxidase-antiperoxidase (PAP) stain, (Sternberger *et a.*, 1970) which uses antiserum specific for the glial acidic fibrillary protein contained in astrocytes (Eng and Bigbee, 1978) and provides a specific immunocytochemical stain for astroglial cells. Areas of interest were photographed with Kodak Ectachrome Professional film (ASA 50) using either a Wild macroscope or Leitz Ortholux microscope.

Biochemistry

Brain Weights and Dissection: Eighteen (TET=6n, starved=6n, saline=6n) randomly selected pups were weighed, decapitated and the whole brain removed and weighed to give relative brain weights among test groups (Table 1). For regional brain weights, the brain was hemisected coronally using the midpoint of the infundibulum as a reference. The anterior brain contained frontal and parietal regions

and the posterior brain included the temporal, entorhinal and occipital components of the cortical mantel.

Receptor Binding Assay: Since perinatal undernutrition is reputed to produce profound effects on CNS neurotransmitters and their receptors (Shoemaker and Bloom, 1977; Telang *et al.*, 1984), we elected to use only body weight-corrected starved controls in the receptor binding assays. PND-20 TET-treated (n=6) and starved-control (n=6) pups were decapitated and the hippocampus removed and dissected according to Iverson and Glowinski (1966) methodology. The pieces were weighed and homogenized in a glass-Teflon homogenizer in 19 volumes 0.32 M sucrose. A total membrane fraction was prepared by centrifugation (20,000 g, 10 min) of a 5% (w/v) tissue homogenate in 0.32 M sucrose. Fractions were stored frozen (−20°C) and membranes were prepared from them by homogenizing precipitates in cold distilled water corresponding to 19 times the original tissue wet weight. Centrifugation (20,000 g, 10 min) was followed by a further homogenization in 40 mM tris-HCl pH 7.4. Protein was estimated by the method of Lowry (Lowry *et al.*, 1951) and membrane suspensions were diluted with tris buffer to a final concentration of 1-2.5 mg/ml.

Quinuclidinyl benzilate (QNB) binding was used to measure muscarinic sites with atropine as a competitor. The binding assay, using 10^{-9}M dl-[benzilic-4, 4'-^{3}H] (Magee *et al.*, 1957) (Ci/mmol New England Nuclear Corp., Boston, MA) was performed by a glass fiber disc filtration method previously described (Bondy, 1982). Saturability, specificity, regional distribution, and characteristics of ligands were delineated prior to this study. Briefly, the method consisted of incubating the washed membranes (100-250 μg protein) together with 10^{-9} M of a tritiated ligand in 40 mM tris HCl pH 7.4 for 15 min at 37°C. The receptor-ligand complex was separated from free ligand by filtration through 0.3μ pore size glass fiber discs (Gelman Inc., Ann Arbor, Michigan) and washed three times with 4 ml Tris buffer. Dried discs were then counted in a scintillation counter (38-43% efficiency) in order to determine the total amount of bound ligand. Nonspecific binding was also determined in a series of parallel incubations in the presence of 10^{-6} M atropine sulphate. Those counts which remained in the presence of this excess of the competitor were taken to represent nonspecific binding.

Benzodiazepine (BZD) receptor sites were similarly estimated with 10^{-9} M [methyl-3H]diazepam (73 Ci/mmol, New England Nuclear Corp., Boston, MA.) and the unlabeled compound at 10^{-6} M was used as a competitor. Incubation was for 15 min at 0°C.

Statistical Analysis: The effect of treatment was evaluated by analysis of variance (ANOVA). This was individually performed for each brain parameter since assays were carried out on separate days. Appropriate pair-wise comparisons were performed with a Fisher's Least Significant Difference (LSD) Test. Acceptable statistical significance was established as $P < 0.05$.

RESULTS

Macroscopic examination: Grossly, the brains of both saline and starved controls were indistinguishable from each other but differed from the tin-treated pups. There was a highly variable response within litters to TET with some brains more severely affected macroscopically than others. The whole brains of severely affected TET-treated pups were characterized by a cavitated ventrolateral surface (Fig. 1). Focal areas of petechial hemorrhages were seen along the meninges of fresh brains and were especially prominent in the posterior

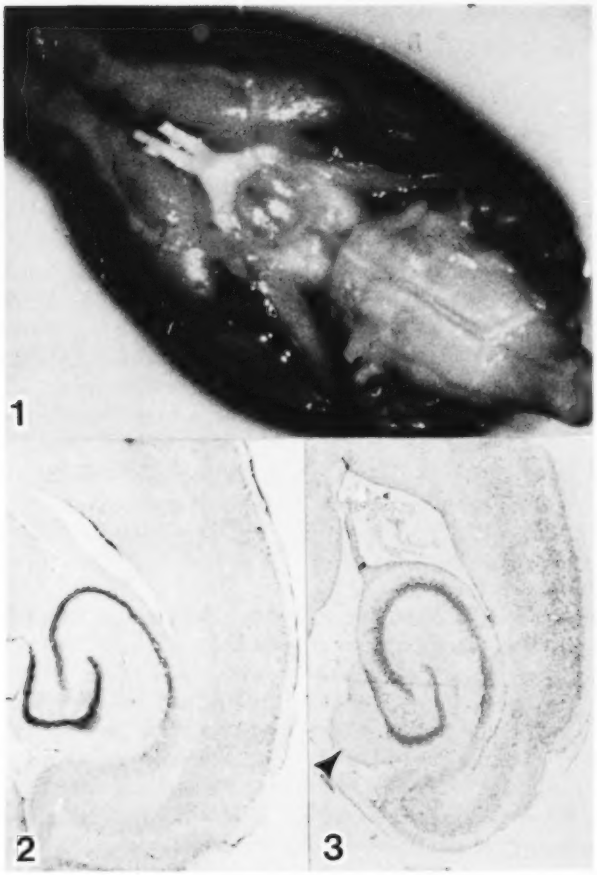

FIG 1. Whole brain of severely affected TET-treated rat showing cavitated ventral-lateral surface. X3.5
FIG 2. Cresyl violet-stained paraffin section of starved control showing normal cytology of the posterior brain. X24
FIG 3. TET-treated cresyl violet-stained section of posterior cortex of TET-treated pup showing compacted laminae of the entorhinal cortex and parasubiculum. Note protrusion of molecular layer in dentate gyrus (arrow). X24

region. When examined in the horizontal plane, the lateral ventricles appeared moderately distended.

Microscopic Examination: Histologically, saline and starved-control tissues showed the well-defined laminated cortex described elsewhere (Swanson, 1979). In these animals, the ERC, located adjacent to the rhinal fissure had a more defined cellular lamination than its neighboring para- and presubicular regions, which were marked by a dense fiber plexus and a compressed layer of granular cells (Fig. 2). The subiculum lacked this laminated appearance and its cells appeared scattered in an apparently unsystematic fashion.

The histopathology of TET-treated animals, was generally confined to the posterior brain, specifically the transitional cortex (i.e., the pre- and parasubiculum), and the hippocampal complex. In contrast to control tissues, where the highly vascular pia mater closely outlined the cortical contour, the meninges of TET-treated animals were retracted from the cortex and appeared

swollen, hemorrhagic and infiltrated with darkly stained cells. There appeared to be a direct relationship between regions of meningial disruption and areas of cortical necrosis. In passing from the pial surface, the molecular layer appeared irregular in width, cell-infiltrated and extremely basophilic. In general, the ERC's lamination pattern appeared severely compressed (Fig. 3).

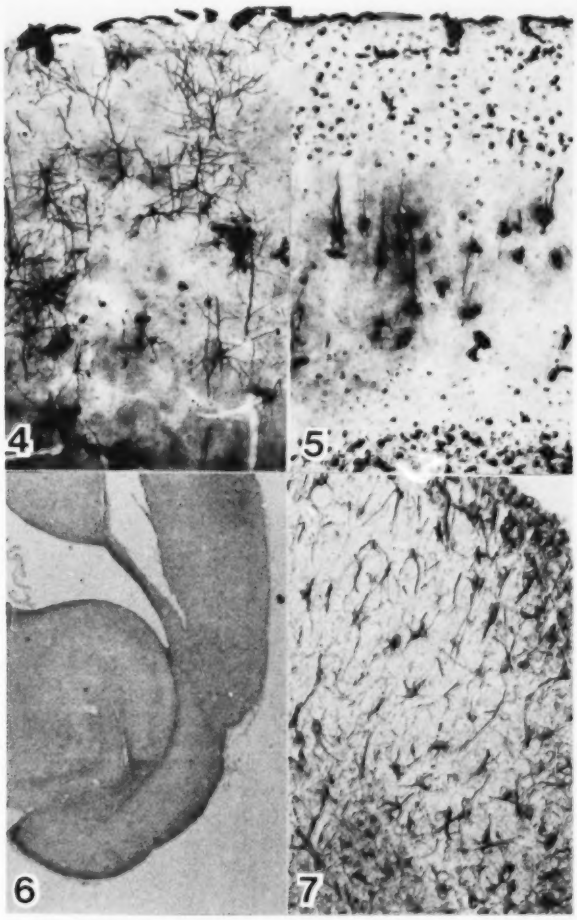

FIG 4. Golgi-stained material of saline-treated posterior cortex showing neurons with well-developed apical and basilar dendritic arborization. X24

FIG 5. Golgi-stained section of TET-treated animal showing necrosis of the upper level neurons and severely withered dendritic arborization in the surviving population of pyramidal neurons. X24

FIG 6. Horizontal section of TET-treated pup brain immunocytochemically stained for astrocytes indicated a replacement of outer-layer neurons of the entorhinal and transitional cortex with astroglial cells. X40

FIG 7. Higher magnification of cortical tissue stained by the PAP technique which imparts a rose-red color to the astrocytic processes and the cytoplasm of their cell bodies. X820

Below the molecular layer, evidence of severe cortical necrosis of the outer layer (II, III) neurons was present. The cell bodies of the inner cortical layers (i.e., the internal pyramidal layer), which consisted of medium and large pyramidal neurons, appeared largely intact although occasional aggregates of microglial were noted. Golgi-impregnated material revealed these inner-level neurons to have severely withered dendritic arborization in contrast to the well developed apical and basilar dendrites seen in saline and starved control tissues (Figs. 4, 5). The upper cellular layers of the ERC and transitional cortex of TET-treated brains were replaced largely by astrocytes (Fig. 6). Astrocytes stained by the PAP immunocytochemical technique developed a rose-red color to their fibrillary structures and were easily identified by light micros-

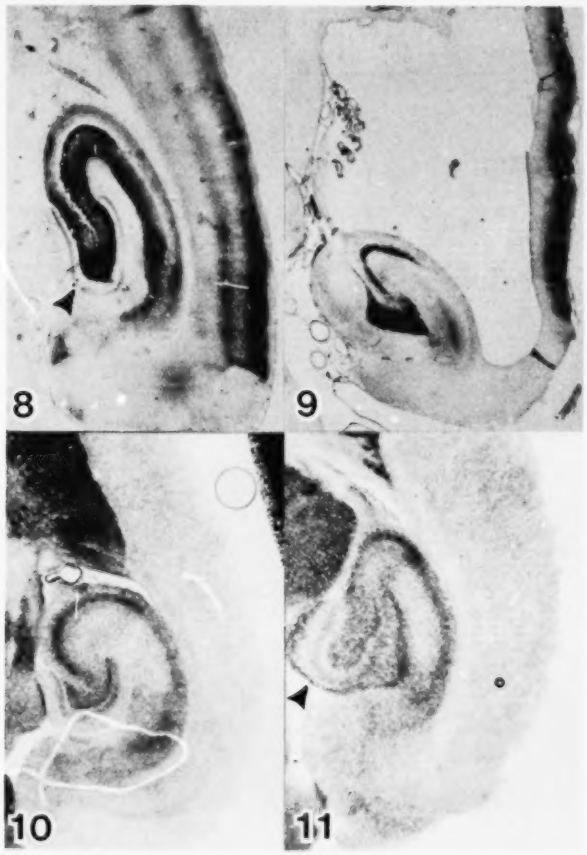

FIG 8. Timm's stain of starved-control tissue showing heavy metal deposits of the entorhinal and the CA4-2 hippocampal complex. Note laminae formation (arrow) around the dentate gyrus. X24

FIG 9. Timm's stain of severely affected TET-treated animal showing restricted staining in the hippocampal proper and complete loss of laminae staining. X24

FIG 10. Starved-control section of posterior brain stained for acetylcholinesterase showing normal pattern of precipitate. X24

FIG 11. TET-treated section showing intense band (arrow) of acetylcholinesterase staining on the outer rim of the molecular layer of the dentate gyrus. X24

copy (Fig. 7).

In both saline and starved control animals, sections stained by Timm's method showed a heavy precipitation in the ERC, dentate gyrus, and the hippocampus proper extending from CA4,3, and 2. Distinct laminae, corresponding to the terminal fields of various afferent systems (Zimmer, 1973), were especially prominant in the molecular layer of control tissues (Fig. 8). Sections from severely affected TET-treated pups, sampled at the same horizontal level as controls and processed identically for the Timm's stain, showed a marked reduction in the staining of the CA4,3, and 2 sectors, with these regions achieving only a fraction of their normal width. A conspicuous absence of those laminae which identified the perforant path zones in the outer parts of the dentate molecular layer of control tissues was also evident (Fig 9).

Control sections of the posterior cortex, stained for AChE, showed precipitate localized in discrete layers of the hippocampus and dentate gyrus (Fig. 10). In sections of TET-treated brain, a similar staining pattern was noted and in addition, there occurred an intense band of AChE precipitation in the outer rim of the molecular layer of the dentate gyrus, an area which gave only a slight reaction in control material (Fig. 11).

The major myelinated tracts of the posterior cortex (e.g., external, internal capsule, fimbria) were examined in the horizontal plane. Delayed myelination was suggested by an ambundance of oligodendroglia with round, pale nuclei as opposed to the mature oligodendrocytes with small, dark, disc-like nuclei arranged in rows within the myelinated tracts. The effects of neonatal exposure to TET on myelin will be discussed in a companion paper.

TABLE 1. Brain Weights.

	BODY AND BRAIN WEIGHTS (gm)		
Group	Whole Body Wt.		Brain Wt.
Saline	46.7 + 1.9		1.42 + 0.03
Starved	*34.0 + 4.2		1.38 + 0.02
TET	*31.9 + 0.9		*1.10 + 0.04
	BRAIN REGIONAL WEIGHTS (mg)		
	Anterior Cortex	Posterior Cortex	Hippocampus
Starved	283 + 37	410 + 15	99 + 4
TET	*199 + 20	*242 + 9	*74 + 9

Body and brain weight data derived from 18 pups (n=6/group). Brain regional weights are expressed as the mean of n=6+SE. (*) indicates TET value is statistically different from corresponding starved or saline value ($p < 0.05$), Fishers least significance test for body and whole brain weights, students' 2-tailed t-test for regional weights.

Brain weights: Table 1 indicates that although the body weights of TET-treated pups did not differ significantly from starved controls, their brains (including olfactory bulbs) weighed approximately 80% of starved and 77% of saline controls. In addition, the regional brain weights of TET-treated pups were significantly lower than the starved controls, the magnitude of this loss being greatest in the posterior cortex.

Receptor Binding Assay: The status of cholinergic circuity was examined in the hippocampus using [3H]-quinuclidinyl benzilate (QNB) as a muscarinic cholinergic receptor ligand. QNB binding was depressed on a per-region basis in TET-treated

TABLE 2. Receptor Binding Assays.

	QNB		Diazepam	
Group	/5 mg tissue/region		P moles bound 5/mg tissue/region	
Starved	0.254 + .007	5.64 + .38	0.177 + .013	4.09 + .47
TET	0.264 + .011	*4.05 + .26	*0.143 + .005	*2.20 + .12

Values are means from 6n animals + SE. (*)=$p < 0.05$ that TET values differ from corresponding starved value. ($p < 0.05$, Students, t-test)

rats but was identical to starved control values on a tissue-weight basis. Hippocampal benzodiazepine receptor sites were also assayed using [3H]-diazepam. Binding capacity was reduced both on a per tissue basis and on a per unit wet-weight basis (Table 2).

DISCUSSION

The present study provides evidence that neonatal exposure to an acute dose of TET results in damage to neuronal populations of the ERC and transitional cortex and a derangement of hippocampal circuitry. This is in line with the development status of the entorhinal-hippocampal circuitry of the altricial rodent at PND-5, a time when the processes of hippocampal neurogenesis (Altman and Bayer, 1970) and more specifically synaptogenesis (Gall and Lynch, 1980; Steward and Scoville, 1976) are largely unfinished, rendering these events especially vulnerable to neurotoxic insult. In normal hippocampal development, major afferents arrive in the dentate gyrus during the first week of life and quickly restrict to their appropriate levels on the granule cell dendrites. These fibers, originating from the ipsilateral ERC cortex and the pyramidal cells in both the ipsi- and contralateral Ammon's horn, terminate in the molecular layer on the granule cells' dendrites in a strictly ordered fashion, with the entorhinal afferents synapsing on the distal two-thirds of the dendrites (Hjorth-Simonsen and Jeune, 1972) and the commissural (contralateral) and associational (ipsilateral) fibers terminating on the proximal one-third (Zimmer, 1971; Gottlieb and Cowan, 1972). A third afferent fiber system, the cholinergic septohippocampal tract, terminates in a stratum between the granule cell bodies and the zone of the commissural fiber synapses (Matthews *et al.*, 1974; Raisman *et al.*, 1965). Synaptogenesis in this region is most active between PND 4-11, a time period when the number of synapses doubles every day (Crain *et al.*, 1973). In view of tin's exposure time and its 7.3d half-life (Cook *et al.*, 1984), synaptogenesis might be expected to be severely affected and may actually represent the crucial target-event in the neonatal model of TET-encephalopathy.

Disruption of hippocampal synaptogenesis in the immature rat has been achieved experimentally by surgical ablation of the ERC, a procedure which results in vacant synaptic fields in the molecular layer of the dentate gyrus. These sites are avidly filled by commissural, septohippocampal and associational fibers which travel beyond their usual proximal placement on the dendritic tree and extend toward the distal surface of the molecular layer to occupy the entire height of the granule cell's dendritic tree (Lynch *et al.*, 1973; Gall and Lynch, 1980; Zimmer, 1973). Pertinent to our data is the histochemical demonstration of AChE-containing septohippocampal terminals in the outer rim of the molecular layer, a location normally reserved for the entorhinal afferents. This staining pattern has been produced by others in surgically de-entorhinalized rat neonates (Lynch *et al.*, 1973; Cotman *et al.*, 1973) and reflects the aberrant hippocampal circuitry produced by the excessive axonal sprouting of the cholinergic fibers. Additional evidence of perforant path disruption comes from the Timm's stain for heavy metal deposition which not only delimits the granule cells' mossy fibers but acts as a potential marker for synaptic fields of the laminated afferent zones of the hippocampus and dentate gyrus. The differential staining pattern seen in TET-treated pups compared to controls is reminiscent of that seen in surgically de-entorhinalized neonates (Zimmer, 1973) and indicates an absence of perforant synapses on the zones normally occupied by terminals from the lateral and medial perforant paths of the dentate gyrus.

Tin's persistance in the juvenile brain (Cook *et al.*, 1984) impacts on other critical events namely hippocampal neurogenesis (Altman and Bayer, 1970) and myeli-

nogenesis (Jacobsons, 1963). Insult to these processes was evidenced by the pyknotic granule cells noted two weeks post-exposure and the delayed differentiation of the oligodendroglial populations. In view of these data, the overall definition of TET neonatal-neurotoxicity should be expanded to include not only the effects of synaptic plasticity and reorganization of the hippocampal circuitry which were suggested by the histochemical and biochemical data, but also the consequences of neuronal and myelin loss.

In view of the critical interdependence that exists between pre- and post-synaptic elements in their development and maintenance (Hamori, 1973), the diminished or absent entorhinal input could expectedly result in an aberrant dendritic arborization of the hippocampal granule and pyramidal neurons. Evidence of dendritic involvement was indicated by the benodiazepine receptor binding data of Table 2. The benzodiazepine receptor has been reported to be post-synaptically localized to the somata and dendritic tree of granule cells (Valdes *et al.*, 1982). In our study, benzodiazepine binding was severely and selectively depressed in the hippocampus of TET-treated rats, data which suggests an actual loss of the granule cell population or a disorganization of its dendritic arborization. The muscarinic receptor, which in the hippocampus is largely post-synaptic (Yamamura and Snyder, 1974; Ben-Barak and Dudai, 1979), appeared less affected by TET. Our data report that the binding of [3H]-QNB in the hippocampus was depressed on a per region basis, but not on a tissue weight basis. This indicated that QNB receptor proteins were not deficient relative to the total protein in this region, an apparent normalcy that may reflect on a down-regulation in response to the increased cholinergic input to the dentate gyrus.

In summary, we have demonstrated that neonatal exposure to TET severely damages the ERC and transitional cortical neurons and disrupts synaptogenesis in the afferent layer of the dentate gyrus. Because of the developmental status of this model, it can be argued that this damage is a composite of both the direct neurotoxic effects of the alkyl tin and the consequences of interrupted or aborted developmental events. In either case, the described changes represent permanent alterations in the neuronal circuitry of the posterior brain and may underlie the reported persistent abnormalities associated with this model.

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